

## No association between HPV infection and the neoplastic progression of esophageal squamous cell carcinoma: Result from a cross-sectional study in a high-risk region of China

Guo-Fu Gao<sup>1</sup>, Mark J. Roth<sup>2</sup>, Wen-Qiang Wei<sup>1</sup>, Christian C. Abnet<sup>2</sup>, Feng Chen<sup>1</sup>, Ning Lu<sup>1</sup>, Fang-Hui Zhao<sup>1</sup>, Xin-Qing Li<sup>1</sup>, Guo-Qing Wang<sup>1</sup>, Philip R. Taylor<sup>3</sup>, Qin-Jing Pan<sup>1</sup>, Wen Chen<sup>1</sup>, Sanford M. Dawsey<sup>2\*</sup> and You-Lin Qiao<sup>1\*</sup>

<sup>1</sup>Department of Cancer Epidemiology, Cancer Institute, Chinese Academy of Medical Sciences, Beijing, People's Republic of China

<sup>2</sup>Nutritional Epidemiology Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, MD

<sup>3</sup>Genetic Epidemiology Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, Bethesda, MD

Esophageal cancer is a leading cause of cancer death, especially in developing countries. In high-risk regions, squamous cell carcinoma is the most common type of esophageal cancer, and its etiology remains poorly understood. The purpose of this study was to evaluate the association between human papillomavirus (HPV) infection and esophageal squamous cell carcinoma (ESCC) and related precursor lesions in a high-risk area of China. We conducted a cross-sectional study among adult inhabitants of Linxian, China. All subjects were interviewed about potential risk factors, had the length of their esophagus sampled by a balloon cytology examination and underwent endoscopy with mucosal iodine staining and biopsy of all unstained lesions. A multivalent HPV hybridization probe, Digene Hybrid Capture II (Gaithersburg, MD), which recognizes high-risk types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68, was used to determine the HPV infection status of the cytologic specimens, and the endoscopic biopsies were used to classify each subject's esophageal disease. 740 subjects completed the cytologic and endoscopic exams, and 702 had adequate cytologic and biopsy specimens. Using a cutpoint of  $\geq 3.0$  pg/ml of HPV DNA to define a positive test, HPV positivity was identified in 13% (61/475) of subjects without squamous dysplasia, 8% (8/102) with mild dysplasia, 7% (6/83) with moderate dysplasia, 16% (6/38) with severe dysplasia and zero (0/4) with invasive ESCC. Changing the cutpoint defining a positive test did not change the association of HPV infection and dysplasia grade. In this high-risk population, infection of esophageal cells with high-risk HPV types occurs in 13% of asymptomatic adults with no evidence of squamous dysplasia and a similar proportion of individuals with mild, moderate or severe dysplasia. This suggests that HPV infection is not a major risk factor for ESCC in this high-risk Chinese population. Further studies are warranted to determine if infection with this agent is associated with neoplastic progression in a subset of cases.

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**Key words:** esophageal squamous cell carcinoma; HPV; Hybrid Capture II

Esophageal cancer is the 6th leading cause of cancer death worldwide, with ~462,000 new cases and 386,000 deaths each year.<sup>1</sup> Over 80% of esophageal cancers occur in developing countries, and most are of the squamous cell type.<sup>1</sup> The etiology of esophageal squamous cell carcinoma (ESCC) in high-risk regions remains poorly understood. Tobacco and alcohol, the dominant risk factors in low-risk countries, are not major determinants of ESCC in most high-risk populations.<sup>2,3</sup> Low consumption of fruits and vegetables,<sup>2,4</sup> micronutrient deficiencies<sup>5–7</sup> low socioeconomic status<sup>2,3</sup> and family history of ESCC<sup>3,8,9</sup> appear to be the most consistent contributing factors in these populations. Poor oral hygiene,<sup>10</sup> polycyclic aromatic hydrocarbon exposure unrelated to tobacco smoke,<sup>11,12</sup> drinking hot liquids,<sup>4,13,14</sup> drinking mate tea<sup>4</sup> and exposure to fungal toxins<sup>15</sup> may also play a role, and other unknown factors may also be important.

It is now widely accepted that nearly all cases of cervical cancer are caused by infection with high-risk types of human papillomavirus (HPV),<sup>16</sup> and an etiologic role for HPV has also been established for subsets of cancers in the vulva, penis, anus, oral cavity and pharynx.<sup>17</sup> In the cervix, HPV infection results in the develop-

ment of squamous dysplasia and its progression to invasive disease, and this progression appears morphologically similar to the neoplastic progression of squamous lesions observed in the esophagus. In 1982, Syrjanen *et al.* first suggested that HPV might be an etiologic factor in ESCC,<sup>18</sup> and many studies over the past 2 decades have examined this topic, with varying results (reviewed in Ref. 19). In part, this variability stems from different HPV detection methods [serology, *in situ* hybridization (ISH), Hybrid Capture, polymerase chain reaction (PCR)], interlaboratory variation performing the same detection method, different methods of diagnosing current or future disease (cytology, endoscopic biopsy, resection), and regional differences. High-risk areas for esophageal cancer, such as China<sup>20</sup> and Iran,<sup>21</sup> tend to report higher rates of HPV infection in ESCC tumors than low-risk areas, such as North America<sup>22</sup> and Europe.<sup>23,24</sup> Even within individual geographic areas, however, results have been quite variable. For example, in the high-risk areas of China, Cao *et al.* found that 78% of their ESCC tumors were positive for HPV,<sup>25</sup> while Chang *et al.* found 49% positive in 1 study<sup>26</sup> but only 17% positive in another study,<sup>27</sup> and Lu *et al.* found no evidence of HPV in tumors from the same population.<sup>28</sup> How HPV infection relates to preneoplastic lesions of ESCC has been much less studied, and is even less well understood.

Linxian, in Henan Province, China, is part of the “central Asian esophageal cancer belt” and, with annual mortality rates of up to 99 per 100,000,<sup>29</sup> is among the highest risk areas for ESCC in the world. Squamous dysplasia is the histologic precursor of ESCC in Linxian, and increasing grades of dysplasia are associated with dramatically increasing risk of ESCC in this population.<sup>30</sup> To evaluate whether an association exists between HPV infection and ESCC in this high-risk region, we used the Digene Hybrid Capture II test to analyze esophageal balloon cytology samples for the most common high-risk HPV types in patients with an esophageal histologic spectrum from normal to 3 grades of dysplasia to early invasive ESCC.

### Material and methods

#### Study subjects

In 2002, as part of a Chinese–American collaboration to study early detection methods for esophageal cancer, a cytologic and en-

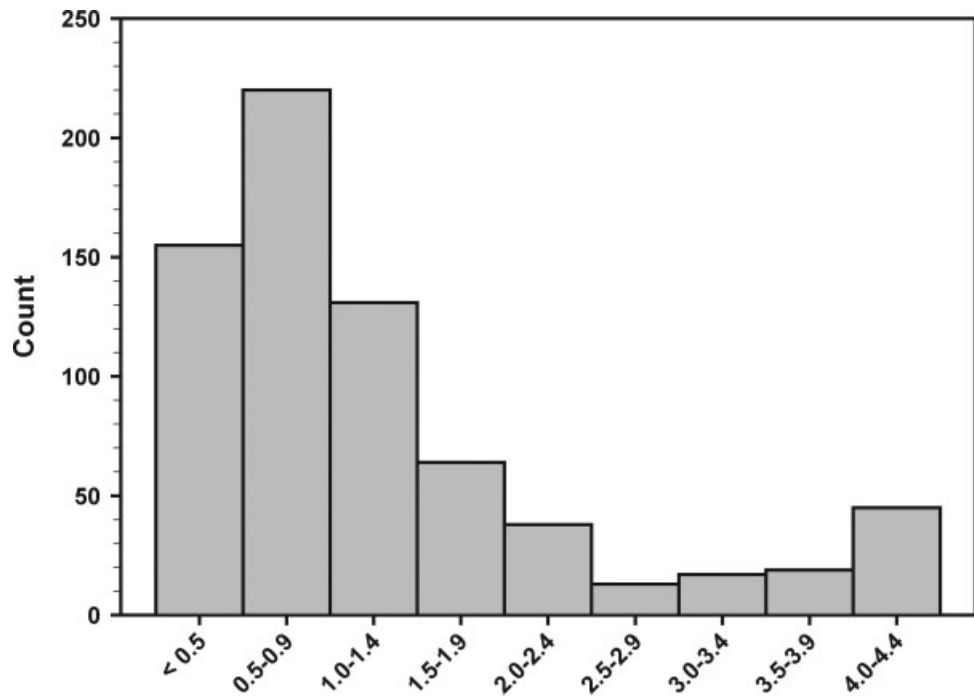
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\*Correspondence to: Cancer Institute, Chinese Academy of Medical Sciences, 17 South Panjiayuan Lane, Chaoyang District, Beijing 100021, PRC. Fax: +86-10-6771-3648. E-mail: qiao@public.bta.net.cn or Nutritional Epidemiology Branch, Division of Cancer Epidemiology and Genetics, National Cancer Institute, 6120 Executive Boulevard, Suite 320, Bethesda, MD 20892-7232, USA. Fax: +301-496-6829. E-mail: dawseys@mail.nih.gov.

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**FIGURE 1** – Distribution of Hybrid Capture II results in the analytic cohort ( $n = 717$ ). Aliquots of esophageal balloon cell samples were assayed using Hybrid Capture II. Results were expressed in pg/ml of HPV DNA.

doscopy screening survey was conducted in 3 villages of Linxian. This study was approved by the Institutional Review Boards of the Cancer Institute of the Chinese Academy of Medical Sciences and the US National Cancer Institute.

Asymptomatic residents from 40 to 65 years of age with no contraindications to esophageal cytology or endoscopy examinations were recruited as subjects. Eligible subjects who agreed to participate and signed an informed consent were assigned a study identification number and referred for the examinations. Each subject was interviewed for demographic and risk factor information, underwent an esophageal balloon cytology examination, and had an endoscopy examination with mucosal iodine staining. The “gold standard” diagnosis for each subject was the worst squamous histology found in the biopsies of the endoscopy exam.

Demographic and risk factor information was gathered on gender, age, cigarette smoking, alcohol intake, family monthly income, education level, and height and weight, which was used to calculate body mass index (BMI). BMI was categorized according to the International Obesity Task Force (IOTF) for Asia, as underweight ( $<18.5$ ), normal ( $18.5$ – $22.9$ ), at-risk for obesity ( $23.0$ – $24.9$ ), or obese ( $\geq 25.0$ ).<sup>31</sup>

#### *Collection and preparation of esophageal balloon cytology specimens*

Following removal of any dental prostheses, a vigorous mouth rinse with water and local anesthesia (benzocaine spray or a lidocaine slurry), the esophageal balloon sampler was passed down the esophagus into the stomach. When the balloon reached the stomach, it was inflated and brought back up the esophagus, collecting exfoliated and scraped surface cells. At the upper esophageal sphincter, the balloon was deflated, to limit oropharyngeal contamination, and removed.

Each balloon was then cut from its tubing, submerged in a 50-ml centrifuge tube with 40 ml of normal saline, and mechanically vortexed to displace adherent cells from the balloon into the solution. The balloon was then discarded. The cell samples were centrifuged, the supernatant was discarded and the cell pellet was resuspended in 1 ml of residual saline. Half of the cell suspension was removed and frozen at  $-80^{\circ}\text{C}$ , and the rest was resuspended in 12 ml CytoRich preservative (Tripath Imaging, Burlington,

NC) and brought back to Beijing. The cellularity of the preserved cell samples was evaluated for adequacy by the criteria of the Bethesda System,<sup>32</sup> and only cell samples with adequate cellularity were tested for HPV DNA.

#### *Collection, preparation and diagnosis of esophageal endoscopic biopsies*

The patients were given a 1% lidocaine slurry for local anesthesia 2–5 min before endoscopy. The endoscope was inserted and the entire esophagus and stomach were examined before and after spraying of the esophagus with 1.2% Lugol’s iodine solution. Biopsies were taken from all visible lesions, including all unstained lesions in the esophagus, and from standard sites in the midesophagus and cardia.

Biopsies were oriented on filter paper fixed in 80% ethanol, embedded in paraffin, and cut and stained with hematoxylin and eosin. The slides were read independently by 2 gastrointestinal pathologists who were blinded to the HPV DNA results, using criteria previously described,<sup>30</sup> and discrepant diagnoses were resolved by joint review. Each patient’s worst esophageal squamous diagnosis was determined using the following hierarchy: invasive ESCC > severe squamous dysplasia > moderate squamous dysplasia > mild squamous dysplasia > no dysplasia.

#### *Hybrid Capture II analysis for the detection of HPV DNA*

Hybrid Capture II (HC2) (Digene, Gaithersburg, MD) is a nucleic acid hybridization assay utilizing microplate chemiluminescence signal amplification to detect 13 high-risk HPV types, including types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59 and 68.<sup>33</sup> The assay results are expressed in relative light units (RLUs), which are the light units found in the specimen relative to the mean of the light units found in positive control specimens containing 1.0 pg/ml of HPV DNA. This assay provided sensitivity and specificity of 94 and 85%, respectively, for identifying CIN2 or higher grade lesions in 8497 subjects in the SPOCCSII cervical cancer screening study in China.<sup>34</sup> Assays were done in Beijing, in the same lab that performed the tests for the SPOCCSII study, and were performed according to the manufacturer’s instructions. To ensure that the cell samples were not contaminated in the lab, all specimen tubes were covered at all times, fresh

**TABLE I** – THE ASSOCIATION OF HPV INFECTION AND DEMOGRAPHIC AND ENVIRONMENTAL VARIABLES, USING AN HPV POSITIVITY CUTOFF OF  $\geq 3.0$  pg/ml

	<i>N</i> (%)	HPV+ (%)	OR <sup>1</sup>	95% CI	<i>p</i> for trend
Sex					
Male	310 (43)	33 (11)	1	Ref	
Female	407 (57)	49 (12)	1.15	(0.72–1.83)	
Age (years)					
40–49	49 (7)	4 (8)	1	Ref	
50–54	315 (44)	36 (11)	1.50	(0.51–4.43)	0.472
55–59	209 (29)	24 (11)	1.51	(0.50–4.59)	
60–65	144 (20)	18 (13)	1.69	(0.54–4.33)	
Family history of ESCC					
No	394 (55)	44 (11)	1	Ref	
Yes	323 (45)	38 (12)	1.07	(0.67–1.69)	
Education					
Illiteracy	70 (10)	11 (16)	1	Ref	
Primary school	541 (75)	61 (11)	0.69	(0.34–1.41)	0.272
$\geq$ Middle school	106 (15)	10 (9)	0.58	(0.22–1.50)	
Family monthly income (\$)					
$<60$	146 (20)	20 (14)	1	Ref	
60–119	251 (35)	30 (12)	0.83	(0.45–1.54)	
120–179	215 (30)	23 (11)	0.73	(0.38–1.40)	0.166
$\geq 180$	105 (15)	9 (9)	0.58	(0.25–1.32)	
Cigarette smoking					
No	536 (75)	64 (12)	1	Ref	
Yes	181 (25)	18 (10)	0.84	(0.41–1.71)	
Alcohol consumption					
No	687 (96)	76 (11)	1	Ref	
Yes	30 (4)	6 (20)	2.31	(0.87–6.10)	
Body mass index (kg/m <sup>2</sup> ) <sup>2</sup>					
$<18.5$	19 (3)	2 (11)	0.67	(0.15–3.06)	
18.5–22.9	311 (43)	31 (10)	0.63	(0.36–1.10)	0.176
23.0–24.9	201 (28)	21 (10)	0.66	(0.36–1.21)	
$\geq 25.0$	185 (26)	27 (15)	1	Ref	

<sup>1</sup>Adjusted for sex. <sup>2</sup>One subject excluded because of missing data.

wash buffer was prepared just prior to each use, and the laboratory was cleaned with disinfectant and sterilized with ultraviolet light daily. Two-hundred microliters of each fixed balloon sample was denatured, and a quarter of the denatured sample was used for each assay. Positive and negative controls were included in triplicate on each assay plate. All negative controls (total  $n = 41$ ) gave signals  $\leq 0.48$  pg/ml, and the CV of all positive controls (total  $n = 47$ ) was 14%.

#### Data analysis

The HC2 data was analyzed at 3 thresholds for a positive test,  $\geq 1.0$ ,  $\geq 2.0$  and  $\geq 3.0$  pg/ml. The manufacturer's suggested threshold is  $\geq 1.0$  pg/ml, but recent studies have suggested that a higher cutpoint may be more appropriate for cervical cancer screening,<sup>35</sup> and our most recent cervical study in China suggested that  $\geq 3.0$  pg/ml was the best cutpoint when screening for  $\geq$ CIN 2 lesions.<sup>34</sup> The distribution of the HC2 results in the current study (Fig. 1) also suggests that  $\geq 3.0$  pg/ml is a better discriminator in our data. This may be because of the large volume of cellular material present in the balloon samples, which may have affected the RLUs emitted in the HC2 test.<sup>35</sup>

Subjects were classified as smokers if they reported ever smoking cigarettes regularly for 6 months or longer. Subjects were classified as drinking alcohol if they reported drinking beer or wine more than a few times per month or if they reported drinking spirits more than rarely or never.

Statistical tests were performed in SAS (SAS Institute, Cary, NC). Logistic Regression was used to examine the association between HPV positivity and demographic factors, environmental factors and histologic diagnoses. Multiple logistic regression was used to calculate adjusted odds ratios. All  $p$  values are from 2-sided tests, and significance was defined as  $p < 0.05$ .

#### Results

Seven-hundred and forty subjects completed the cytologic and endoscopic exams. Twenty-three of these subjects had cytologic samples with inadequate cellularity and were not tested for HPV, leaving 717 subjects in the analytic cohort. These subjects had a male to female ratio of 1:1.3, and an average age of 55 years. Among them, 25% were smokers, 4% consumed alcohol and 85% completed primary school or less education (Table I). The distribution of HC2 results is shown in Figure 1. Using the cutpoint of  $\geq 3.0$  pg/ml to define HPV infection, there was no significant association between HPV infection and any of the covariates (Table I). The results were similar using the other cutpoints for HPV positivity (data not shown).

Fifteen subjects in the analytic cohort had inadequate endoscopic biopsies, so 702 had data available to evaluate the association of HPV infection and histologic disease. The distribution of worst squamous biopsy diagnoses included 68% with no evidence of dysplasia, 15% with mild dysplasia, 12% with moderate dysplasia, 5% with severe dysplasia and  $<1.0\%$  with invasive ESCC (Table II). At each positivity cutpoint, there were similar proportions of HPV-positive individuals in each of the histologic strata (Table II). Using the  $\geq 3.0$  pg/ml cutoff, no association was identified between HPV infection and any grade of squamous dysplasia, with or without adjustment for covariates (Table III), and this was also true using the other cutpoints for HPV positivity (data not shown).

#### Discussion

The current study used the Digene Hybrid Capture II test to identify high-risk HPV infection in esophageal balloon cell samples from asymptomatic inhabitants of one of the world's highest risk regions for esophageal squamous cell carcinoma. Each partic-

TABLE II – HPV INFECTION BY WORST SQUAMOUS BIOPSY DIAGNOSIS, USING DIFFERENT HPV POSITIVITY CUTPOINTS

Worst histologic diagnosis	No. of patients <sup>1</sup>	≥1.0 pg/ml (%)	≥2.0 pg/ml (%)	≥3.0 pg/ml (%)
No dysplasia	475	217 (46)	95 (20)	61 (13)
Mild dysplasia	102	48 (47)	15 (15)	8 (8)
Moderate dysplasia	83	39 (47)	13 (16)	6 (7)
Severe dysplasia	38	21 (55)	9 (24)	6 (16)
ESCC	4	2 (50)	0 (0)	0 (0)
Total	702	327 (47)	132 (19)	81 (12)

<sup>1</sup>Fifteen subjects excluded because of missing data.

TABLE III – CRUDE AND ADJUSTED ODDS RATIOS AND 95% CONFIDENCE INTERVALS FOR HPV INFECTION BY WORST SQUAMOUS BIOPSY DIAGNOSIS, USING AN HPV POSITIVITY CUTOFF OF ≥3.0 pg/ml

Worst histologic diagnosis	Crude OR	95% CI	Adjusted OR <sup>1</sup>	95% CI
No dysplasia	1	(Ref)	1	Ref
Mild dysplasia	0.58	(0.27–1.25)	0.57	(0.26–1.24)
Moderate dysplasia	0.53	(0.22–1.27)	0.56	(0.23–1.36)
Severe dysplasia	1.27	(0.51–3.17)	1.37	(0.54–3.47)
ESCC <sup>2</sup>	–	–	–	–

<sup>1</sup>Adjusted for sex, age, family history, education, income, smoking, drinking and BMI. <sup>2</sup>Number of patient with ESCC was 0; therefore, corresponding ORs/CIs were not available.

ipant underwent upper endoscopy with mucosal iodine staining and biopsy of all unstained lesions, a highly sensitive method for detecting squamous dysplasia and early ESCC,<sup>36</sup> and was categorized by their worst histologic diagnosis. High-risk HPV infection was detected with nearly equal frequency in individuals with histologic diagnoses across the spectrum from normal to severe squamous dysplasia: 13% of individuals with no dysplasia, 8% of those with mild dysplasia, 7% of those with moderate dysplasia, 16% of those with severe dysplasia, and none of those with ESCC were HPV-positive. Changing the HC2 assay cutpoint defining a positive test changed the apparent prevalence of HPV positivity in the study cohort but did not change the association between HPV positivity and dysplasia grade. A previous study has shown that this histologic grading of dysplasia is highly predictive of ESCC risk in this population,<sup>30</sup> so our finding of similar rates of infection in all dysplasia grades suggests that HPV is unlikely to be a major risk factor for ESCC in this area.

Previous studies of the association of HPV and ESCC in high-risk areas of China have found widely varying results. Studies evaluating biopsy or esophagectomy samples by ISH or PCR have reported HPV DNA in 0–35% of normal tissues,<sup>28,37,38</sup> 6–22% of dysplastic tissues<sup>27,39</sup> and 0–78% of tumor samples.<sup>25,28</sup>

There have been only 4 previous studies evaluating HPV infection in esophageal balloon cytology samples from this area of China. Chang *et al.* tested cell samples from 80 patients with filter ISH for HPV types 11, 16 and 18 and found HPV in 22% of those with normal cytology, 72% of those with cytologic dysplasia, and 67% of those with a cytologic diagnosis of ESCC.<sup>40</sup> Peixoto-Guimaraes *et al.*, using PCR with general primers sensitive to a wide spectrum of HPV types, found HPV in 7% of 57 healthy subjects with normal cytology and 6% of 32 patients with biopsy-proven ESCC.<sup>41</sup> Li *et al.* tested balloon cytology samples from 138 volunteers from a high-risk village by ISH and PCR for HPV 16. By ISH, they found HPV in 21% of those with normal cytology, 80% of those with cytologic dysplasia, and 100% of those with a cytologic diagnosis of ESCC. By PCR, these numbers were 60, 84 and 100%, respectively.<sup>42</sup> Cao *et al.*, using PCR with both general and type-specific primers, found HPV in 57% of 357 volunteers without cytologic evidence of malignancy, including 51% with HPV 16 or 18.<sup>25</sup> The current study, using a different HPV detection technique (Hybrid Capture II, which detects 13 high-risk HPV types) and a different method of patient disease categorization (endoscopy with mucosal iodine staining and biopsy, which is more sensitive and specific than esophageal cytology for detecting squa-

mous dysplasia),<sup>43,44</sup> found HPV in 13% of normal subjects and similar percentages of subjects with all grades of dysplasia.

Many of the studies evaluating balloon cytology samples for HPV have found higher infection rates than the studies analyzing esophageal tissue samples. This may in part reflect contamination of the balloons with HPV-positive oral or pharyngeal cells when the balloon is passed into or removed from the esophagus. HPV is not uncommon in the oral cavity, and it has been etiologically linked to a subset of oral and pharyngeal squamous cell carcinomas.<sup>17</sup>

It is apparent that many factors can contribute to the considerable variability of results reported by studies evaluating the association of HPV infection and ESCC, including the specimen analyzed (serum, esophageal tissue, esophageal balloon cell samples), the HPV detection method used (serology, immunohistochemistry, ISH, Hybrid Capture, PCR with general or specific primers that detect different spectra of HPV types),<sup>45</sup> interlaboratory variation in performing the same detection method,<sup>46–48</sup> the method used to categorize patient disease (cytology, biopsies from endoscopy with or without iodine staining, histology from different parts of resection specimens), and the populations evaluated (which can differ by geography, customs, ethnicity and ESCC risk). The fact that this variability is so pronounced, however, in studies of HPV and ESCC, in contrast to studies of HPV and cervical or other HPV-related cancers, where similar possibilities for variation exist, implies that any causative association must be much weaker and/or less common in the esophagus. Prospective studies may be useful to evaluate this association further, and to look for subsets of cases where an association might be evident.

The current study had several strengths, including the use of a highly sensitive and specific HPV detection method, categorization of esophageal disease status by the current gold standard for esophageal diagnosis, and a large number of patients with squamous precursor lesions. The current study was limited by a small number of patients with ESCC, the possibility that the balloon samples could have been contaminated with HPV-positive cells from the oral cavity, the possibility that the balloons did not sample some of the diseased foci in the esophagus, the fact that we did not perform HPV analysis on the lesion biopsies used for histopathologic diagnosis, and the possibility that an etiologically significant HPV type was not included in the Hybrid Capture II panel.

In summary, we evaluated esophageal cell samples from 702 asymptomatic adults from Linxian, China, and found high-risk HPV types in 13% of subjects with no evidence of squamous dysplasia and a similar proportion of individuals with mild, moderate and severe dysplasia. This suggests that HPV infection is not a major risk factor for ESCC in this high-risk population. However, further studies are warranted to determine if infection with this agent is associated with neoplastic progression in a subset of cases.

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